Purification of cellular membranes enriched in CCR5 or CD4 and preparation of the corresponding proteoliposomes

Thus, it is possible to reconstitute receptors in liposomes containing the following, starting from Sf9 cellular membranes:

1. CCR5 only,
2. CD4 only,
3. CCR5 and CD4 in proportions chosen from cells expressing CCR5 and CD4 separately,
4. CCR5 and CD4 in chosen proportions starting from cells expressing CCR5 and CD4 at the same time, and in identical quantities.

The objective is to obtain HIV envelopes fusion with the HIV co-receptor and then to stop this fusion using a binding agent such as paraformaldehyde or glutaraldehyde and to inject this immunizing pair into huCD4/huCCR5 transgenic mice or into macaques or other monkeys. It may then be possible to inject the preparations into man, depending on the results.

The same system is set up for CXCR4.

Strategies for the reconstitution of transmembrane proteins in proteoliposomes

Sf9 cells of *Autographa californica*, that overexpress CCR5 (or CXCR4) and/or CD4 receptors, will be digested by appropriate detergents in order to obtain proteoliposomes using a method derived from Rigaud et al., 1988, Biochemistry, 27, 2677-2688, Paternostre et al., Biochemistry 1988, 27, 2668-2677; Gaymard et al., J. Biol. Chem. 1996, 271, 22863-22870.

Evaluation of the functional capacities of CCR5 and/or CD4

1 - expressed at the surface of Sf9 cells

a) Presence of receptors at the cellular surface analysed by FACS and confocal microscopy:

I. With specific anti-CD4 or anti-CCR5 antibodies,

II. With gp120 marked by specific antibodies,

III. With HIV-1 carrying muted or unmuted envelopes,

IV. Initially, the function of receptors on the surface of Sf9 cells is characterized and the number of molecules per cell for which we know the lipidic environment of cellular cells, is quantified by scatchard (Cahoreau et al above).

b) Specific confocal fluorescence analysis of fusion by methods derived from Robert Blumenthal (NIH, personal communication, 2000) and by Vidal et al.'s methods, 1996, J. Biol. Chem. 270, 17823-17829.

I. After contact with cells expressing the HIV-1 envelope (muted or not muted),

II. After contact with HIV-1 (or viral pseudotypes carrying muted or unmuted envelopes),

III. With viral pseudo-particles.

2-in the corresponding proteoliposomes

a) Specific confocal fluorescence analysis using the above methods

b) Other energy transfer methods (FRET: Fluorescence resonance energy transfer, Mattjus et al., 1999, Anal. Biochem. 268, 297-304).

CCR-5, Introduction of 6 Histidine residues in C-terminal

1 - Amplification by PCR of the C-terminal region between the EcoRI site and the TGA for CCR5:

5' 3'

CCT TCC AGG AAT TCT TTG GCC

Bac-CCR5: add a StuI site (created by degeneration of the genetic code) and an XbaI site into this oligonucleotide, for reintegration of the muted fragment into the original plasmide.

Val	gly	leu	opa
GTG	GGC	TTG	TGA-
CTC	GGA	TTA	
GTA	GGT	CTA	
GTT	GGG	CTG	
		CTC	
		CTT	

StuI XbaI

5' 3'

G GAA ATA TCT GTA GGC CTG TGA CAT CTA GAG GTG

C CTT TAT AGA C AT CCG GAC ACT GTA GAT CTC CAC

3' 5'

matched not matched

The amplified EcoRI-XbaI fragment is cloned in a pUC vector in EcoRI-XbaI and is then sequenced. The muted fragment is then reinserted in the original EcoRI-XbaI plasmide.

2 - Introduction of the 6 histidine codons on the output side of the CCR5 C-terminal

The plasmide thus modified is cut by StuI and Xba and is then bonded with the StuI-XbaI DNA fragment described below. This fragment carries 6 Histidine codons and a Stop TAA codon.

1/2 EcoRI StuI BamHi 1/2XbaI

AA TTC-A GGC CTG CAC-CAT-CAC-CAT-CAT-CAC TAA GGATCC T

G T CCG GAC GTG-GTA-GTC-GTA-GTA-GTC ATT CCTAGG AGATC

An Eco-RI site is added on the input side to clone oligonucleotides matched in an intermediate pUC vector, and thus to verify the sequence.

Modification and cloning of CD4

1 - Sequencing of the C-terminal region of the pGEM-T plasmide containing the CD4 gene:

The C-terminal region of the plasmide is verified by sequencing after a PCR* step.

2 - Addition of 6 histidine residues in the CD4 C-terminal:

1-Amplification of the Bsu361-BanIm region by PCR (in the polylinker)

PCR oligonucleotide:

FOR-CD4:

5' 3'

CCT AAGCTG ATG CTG AGC TTG

BAC-CD4:

BamHi PstI

5' 3'

CAGT GGATCC AAT GGG GCT GCA GGT CTT CTG

2-Addition of 6 His codons

1/2 PstI 1/2 BamHI

GC CCC ATT CAC CAT CAT CAC CAC CAT TTA G

ACGTCG GCG TAA GTG GTA GTA GTG GTG GTA ATT CCTAG